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The most distal region of chromosome 17p between 17p13.3 and the telomere have been found to be independently deleted in several breast tumors. The overall goal of this project is to produce a physical mapping resource which would allow the identification of genes important in breast carcinogenesis. A major obstacle in the way of further study of this region has been the paucity of polymorphic markers localized to it. This problem has been approached by the construction of a microdissection library of this region. In brief, microclones dissected from 17pter were PCR-amplified with degenerate primers (UN1), the product subsequently biotynilated and used in FISH experiments to confirm localization. A second round of amplification led to direct cloning into pAMP UDG vector. After transformation of E. coli with the recombinant plasmids, colonies containing inserts are selected by X-gal blue/white color selection. The microclone library was analyzed by FISH analysis on human metaphase chromosomes and hybridized to Cot-1 DNA. Every Cot-1 negative clone was hybridized to a gridded chromosome 17 cosmid library, and one positive cosmid form each hybridization was identified. The identification of cosmids in this area is an important first step towards the isolation of markers.

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TABLE OF CONTENTS

Cover.....1

Report Documentation Page SF 298.....2

Foreword.....3

Table of Contents.....4

Summary.....5

Specific Aims.....6

Significance and Preliminary Studies.....6

Research Design and Methods.....7

References.....10

Appendix 11

SUMMARY

Germline mutations in breast cancer susceptibility genes account for 8% of all breast cancer cases. *BRCA1* on 17q12 and *BRCA2* on 13q12-13 are implicated in 50%-60% of breast cancer families, and they are both likely to also have an impact in sporadic breast cancer cases. Recently defined loci on chromosome 8p12-22 are frequently lost in sporadic breast cancers and there is evidence for families with linkage to these loci. We propose to ascertain 8 ethnically diverse (non-Caucasian) families linked to putative *BRCA3* loci on chromosome 8p, and to investigate whether a recently isolated prostate cancer metastases suppressor gene, *KAI1*, is involved in the genetic alterations of inflammatory breast cancer, a particularly aggressive form of breast cancer which accounts for 15% of all cases.

1. SPECIFIC AIMS

Hereditary breast cancer accounts for 5-8% of breast cancer cases (1). Loci on 8p12-22 are frequently lost in breast cancer and may thus harbor tumor suppressor genes (2). Almost all of breast cancer families studied so far are of Caucasian origin. *We hypothesize that more ethnically diverse families with evidence of autosomal dominant transmission of breast cancer are needed to study loci on 8p12-22.* As current data indicate that *BRCA2* families are rarer than previously predicted, *we hypothesize that a proportion of families found to be unlinked to both BRCA1 and BRCA2 will be linked to recently defined loci on chromosome 8p12-22; as a corollary, the characterization of the 8p-linked familial tumors will help define the limits of the candidate regions on this chromosome.* The specific aims proposed in this application are:

1.1. To ascertain at least 5 site-specific breast cancer families of diverse ethnic background who are linked to loci on chromosome 8p12-22.

1.2. To analyze the 8p-linked tumors for LOH at closely spaced markers to help refine the breast cancer candidate region(s).

1.3. To test families unlinked to *BRCA1*, *BRCA2*, and loci on 8p12-22 for linkage to the *KAI1* gene, a metastases suppressor gene, and to analyze sporadic breast tumors for mutations in *KAI1*.

2. SIGNIFICANCE AND PRELIMINARY STUDIES

2.a. Family collection. Is it an important part of the study of breast cancer susceptibility genes? Breast cancer will affect 1,600,000 women world-wide in 1995. In the US alone, 48,000 died of this disease (3) in 1994. The discovery of breast cancer susceptibility genes has allowed the study of a relatively simple and unique model system for human mammary epithelial carcinogenesis. *The tumors suffered by the women belonging to these families are clinically indistinguishable from sporadic tumors. Our laboratory has recently proven that at least 10% of sporadic ovarian cancers undergo inactivation of both BRCA1 alleles.*

The scarcity of African American and other minority families in the study of breast cancer susceptibility genes provides the impetus for this application which combines basic research in molecular genetics of breast cancer with a rational attempt at a minority family collection (5-8). The study of the etiology of the widening gap in survival (3) between Caucasian and African American populations requires that future experiments on cancer susceptibility genes be done on an ethnically balanced population of samples. *The lack of availability of these samples and the long lag-time for their acquisition have invariably been the limiting factors. Our proposal intends to contribute shared resources . The Breast Cancer Tissue Core at the University of Michigan (DOD-funded; S. Merajver, co-investigator) provides an independently funded mechanism to freely share these resources for the next 4 years.*

Epidemiologic data on family history and breast cancer incidence on Hispanic, Japanese, and Arab women suggests that familial clustering of breast cancer also appears in these populations (9-11). We have been involved for 5 years in an extensive breast cancer family collection and counseling which was at first dedicated to the positional cloning effort of the breast cancer susceptibility gene, *BRCA1* (12,13). *Since 7/1/94, under the direction of Dr. Sofia Merajver, the Breast Cancer Genetics Project has ascertained 114 new breast cancer families. Thirty of those families are in active collection, and 7 have been completely collected.* The principal sources of referrals are: the University of Michigan Breast Care Center (BCC) (450 new breast cancer patients a year), community surgeons, oncologists, and general practitioners; physicians at other research institutions. We have established a successful collaboration with researchers at Henry Ford Hospital (HFH) in Detroit, MI, where a large number (over 150/year) of African American and Hispanic breast cancer patients with a family history are diagnosed and treated. *The family collection at the University of Michigan has proven invaluable in the localization of BRCA1 and in the assessment of familial mutations. The collection provides a renewable source of RNA and DNA for functional studies after a tumor suppressor gene is cloned. The families ascertained through the research have the option of gaining knowledge of their risk if they so wish under approved protocols.*

2.b. Linkage studies to 8p12-22 and other loci. Is there evidence for a potential *BRCA3* and other tumor suppressor genes on 8p12-22? Why is it reasonable to test families (unlinked to *BRCA1* and *BRCA2*) to this locus? Fueled by the observation in several laboratories that fewer (10-20%) breast cancer families than previously anticipated (40-50%) are linked to *BRCA2*, other candidate loci such as 8p12-22 are being actively tested, where LOH has consistently been observed above background levels. Forty-seven percent of tumor samples have LOH here and at least one breast cancer family with a LOD (logarithm base 10 of the odds favoring linkage) score of 3.70 for markers in this locus has been described. The study of specific breast cancer families linked to the different loci will provide key information and materials for the

understanding of these pathways. *The preliminary studies described here show that there is enough statistical evidence to pursue other tumor suppressor loci as putative familial cancer susceptibility genes on chromosomes 8p12-22 and 11p.*

2.c. Loss of heterozygosity (LOH) Following Knudsen's "two-hit hypothesis" (19), loss of heterozygosity (LOH) is generally a marker of the presence of a tumor suppressor gene. S. Merajver has studied LOH in familial and sporadic breast and ovarian tumors for the last 4 years (4,12). *These experiments demonstrate that LOH at closely spaced, ordered markers on a tumor suppressor candidate region can define a minimum region of overlap and help localize the desired transcript. This experimental design can be extended to other tumor suppressor regions, such as 8p12-22.*

2.c. Mutation analyses of familial and sporadic specimens. Advances in familial cancer syndromes are also relevant to sporadic carcinogenesis. For example, the PI and others have recently shown that *BRCA1* is implicated in sporadic carcinogenesis (4). Dr. Merajver's laboratory was the first to report 4 somatic mutations (12). The initial screen for mutations was performed by a modification of single-strand conformation polymorphism (SSCP) technique. These *experiments show that even for a fairly large gene such as BRCA1, thorough, semi-automated, batch-mode mutation analyses can be performed efficiently and rapidly with a combination of SSCP and direct sequencing of familial and sporadic tumors. Even in the complicated situation posed by BRCA1, these methods have proven effective; we believe that it is not overly optimistic to predict that they will serve us well for tumor suppressor genes on 8p12-22.*

SUMMARY OF PRELIMINARY STUDIES .

The PI has performed family collection studies (13), directs a high-risk breast cancer clinic where family members are counseled, has conducted detailed mapping studies of sporadic tumors by LOH (12), and has ascertained that the mutations of the first described familial breast cancer gene (*BRCA1*) are present in sporadic tumors (4). Research in all these areas has been published in the peer-reviewed literature. (4,12,13)

3. RESEARCH DESIGN AND METHODS

AIM #1. To ascertain at least 5 site-specific breast cancer families of diverse ethnic background who are linked to loci on chromosome 8p. The tasks necessary to accomplish this aim are:

3.1.1. Family ascertainment. We plan an information network by enlisting the cooperation minority clergymen in the metropolitan Detroit area and by an ongoing collaboration with HFH. We will need to screen 30 families to ascertain 5 appropriate kindreds. We seek families with 3 or more cases of female breast cancer in 2 or more generations within a lineage, average age of onset < 50 years, bilateral disease, or male breast cancer. Because of our success in obtaining and using tumor blocks, we are not limited by the number of living affected individuals, as long as we are able to ascertain that the tumor blocks are indeed available. We may ascertain 3-4 8p12-22 families through the screening of new minority cases at UM and HFH. The remaining 2-3 *BRCA2* families will be ascertained through the community-based effort we have begun with African American church leaders in the Detroit metropolitan area, which has a 40% minority population communication. We aim to complete the family ascertainment and analyses of pedigrees during the first year even if the community-oriented efforts are only half as successful as our expectations.

3.1.2. Familial specimen collection. The collection of blood specimens will be undertaken in the manner in which we have proceeded so far. In brief, after signing informed consent, we draw 3 lavender-top tubes (5 cc each) for DNA and 2 green-top tubes (for lymphocyte immortalization). The DNA extraction and immortalization protocols are described below. Pre-paid mailing kits or travel to the family's domicile are used for remote specimens. This latter method is very cost-effective, allows for the establishment of close rapport with the family, and provides for an opportunity to answer questions regarding the research and breast cancer.

3.1.3. Linkage analyses. Linkage analysis is performed using the method of lod scores (14-16). Calculations are carried out with the programs MENDEL (15) and LINKAGE (16). All families will be screened for linkage to *BRCA1* with *D17S855* and *D17S1323*, two intragenic polymorphic markers, and *BRCA2*., the latter with *D13S221, D13S260, D13S267, and D13S263*. Following Kerangueven et al (2), we will use the following panel of markers on 8p12-22 (Table 1).

For each family not linked to either *BRCA1*, or *BRCA2*, or 8p12-22, rapid SSCP analyses of 2 affected individuals with age of onset of cancer under 50 will be conducted on the *KAI1* gene on 11p11.2 (17).

TABLE 1. 8p markers to be used in this study ordered from centromere to telomere

Tumor LOH	Linkage studies
D8S137	D8S137
NEFL	*NEFL*
D8S259	
D8S133	D8S133
D8S258	
LPL	
D8S261	D8S261
D8S265	

3.1.4. Counseling and long-term follow-up of at-risk individuals in a high-risk breast cancer clinic. This *aim has already been accomplished . Dr. Merajver established and is Director of an ongoing 1/2-day-per week High-Risk Breast Cancer Clinic.* Our collaborative experience in the counseling and follow-up of at-risk individuals and of individuals with cancer who belong to breast cancer families is summarized as a guide for clinicians in a recent article (13). The families ascertained through the research proposed here have the services of our High-risk clinic available to them. However, participation in the research is entirely independent from the operations of the clinic; the patients involved in the research need never even meet the investigator(s) and the results of the research are strictly confidential, are not part of the patients medical records, and no charges are incurred by the patients for participation in the research study.

Aim # 2. To analyze the 8p-linked tumors for LOH at closely spaced markers to help refine the breast cancer candidate region(s). The tasks necessary to complete this aim are:

3.2.1 Selection of sporadic and familial specimens. Paraffin-embedded breast tumors families linked to 8p loci will be obtained from the institutions where the patients have undergone surgical treatment, in accordance with guidelines from the University of Michigan Human Use Committee for the procurement of archival materials for research. Living family members will provide informed consent to proceed with the analyses of their own tumors; the results will be shared with the patients if they so wish, under protocol guidelines for counseling families at risk. Next-of-kin will provide authorization to procure specimens from diseased relatives. All specimens will undergo histologic examination to confirm the diagnosis of invasive cancer and to determine whether both neoplastic and normal components are present. All ductal adenocarcinomas will be assessed for Bloom-Richardson grade and all cancers will be evaluated for the degree (percent) of normal stromal infiltration. Blocks with over 75% normal infiltration will not be used for LOH. At most 5% of the tumors will be rejected for this reason.

At the University of Michigan (UM), 450 new patients with breast cancer are seen per year, and approximately 300 of them are newly diagnosed at UM. Our population of new sporadic tumors is 12% African American and 5% is of Hispanic origin. Out of 600 new diagnoses per year HFH, 35% are African American. We expect to fulfill our requirement for sporadic samples for our study from these two sources (UM and HFH).

3.2.2. Archival and fresh specimen collection. All tumor and prophylactic surgery specimens are procured at operation. At the UM, fresh tumor specimens are immediately frozen in liquid nitrogen. Paraffin-embedded tumor blocks for DNA extraction are requested from outside institutions by the same method already in place for the blocks collected for the preliminary LOH experiments described above and for genotyping of diseased individuals. The fresh-frozen tumors will be a valuable source of mRNA for Northern blot analysis of cDNA's and for in situ hybridization studies. The most challenging situation for the analysis of these genes' expression would arise if the gene(s) were only expressed in normal breast and ovarian epithelium so that the transcript from the mutated allele (if any) might only be detected in the normal tissue of mastectomy and oopherectomy specimens from carriers. The fresh-frozen tumor collection will ensure the availability of large amounts of normal tissue which could be used for mRNA extraction, whereas the immortalized blood lymphocytes will provide the matched specimen for DNA extraction and mutation analysis.

3.2.3. DNA extraction of paraffin-embedded tumors. Each paraffin block will be cut in 4 μm sections and mounted on glass slides. Normal and neoplastic tissue fractions will be dissected with a single-use disposable razor blade under a microscope. This procedure minimizes mixing of normal and tumor subpopulations, and yields tumor samples of 1000-10000 cells. The samples will be deparaffinized with 100 μl of xylene. An equal volume of 100% ethanol will be added and the samples pelleted for 10 min. at 15,000g, vacuum-dried, and digested overnight with 200 ng/μl Proteinase K in 100 μl of 50 mM tris, pH

8.3. The samples will be boiled for 8 minutes, iced, and centrifuged again to remove proteinaceous and other debris. This method avoids the use of ionic detergents, sonication, or phenol-chloroform separation, all of which yield samples which may be suboptimal for PCR experiments. If further extractions are needed for a given block, these are carried out on sections immediately adjacent to the original slide; this procedure results in the re-extraction of tumor/normal DNA from regions equivalent to those of the original extraction. All tumors will be dissected and extracted at least twice in separate batches for verification of results.

3.2.4. PCR Amplification of Polymorphic Markers. PCR reactions are performed on each tumor/normal pair, a normal human DNA control, and 2 or more samples from CEPH parents. The reaction volume is 35 µl. The reaction mixture consists of 5-8 µl of DNA template, 3.5 µl 10X Taq polymerase buffer, 4.25 µl of 1.25 mM dNTP mixture, 0.2 µl of Taq polymerase, 0.8-1.5 µl of a 10 ng/µl solution of forward and reverse primer, and the remainder in double-distilled water. One of the primers is end-labeled with ^{32}P-dATP using T_4 kinase. The annealing time is 45-60 seconds whereas the extension time varied from 1 minute to 40 sec + 5 sec/cycle for 35 cycles. Non-specific signals are suppressed by increasing the annealing temperature and/or decreasing the primer concentration. An important requirement for an array of markers to be used in tumor LOH and family linkage analyses is that it be ordered along the chromosome (Table 1).

3.2.5. Lymphocyte immortalization. If a large amount of DNA and a source of RNA are desirable for some experiments, the simplest way to achieve this is by immortalizing each sample with Epstein-Barr (EBV) virus. All samples will be sent to the University of Michigan Clinical Research Center (UM-CRC) facility for lymphocyte immortalization. Lymphocyte immortalization is an uncomplicated technique. Briefly, mononuclear cells are isolated from peripheral blood samples by Ficoll gradient centrifugation. The cells are resuspended in RPMI cell culture media containing cyclosporine to inhibit T-lymphocytes. Monocytes are removed by virtue of their adherence to tissue culture plastic. The B-lymphocytes are then co-cultured with tittered EBV for two weeks. The EBV virion circularizes shortly after entering the cells and replicates as a plasmid. Because the EBV DNA does not integrate into the human genome during the latent phase of infection, the immortalization process should not affect these studies. The immortalized lymphocytes are frozen in liquid nitrogen and used for years as a renewable DNA and RNA resource.

3.2.6. Determination of LOH. We have used the following guidelines to ensure consistency of scoring of LOH. In experiments where the band intensity of the alleles varied between the tumor and normal samples, but the wild-type allele is still visible, the samples are re-extracted from immediately adjacent sections at least 2 times and independent experiments are performed on the tumor-normal pairs. A decrease in intensity of a band of over 50% is required for scoring LOH. The signal intensity will be evaluated visually by at least three independent experienced observers. Because of the presence of at least 2 distinct (possibly 3) loci independently deleted on 8p, we will construct minimum overlap regions for each deletion domain.

We hypothesize that there will be differences in the frequency of interstitial deletions at the 8p and *KAI1* loci between the Caucasian and African American populations. If no differences are found at these loci, we will test for differences at the *BRCA1* locus, where preliminary experiments (Pierce and Merajver, unpublished data) reveal statistically significant differences in the frequency of loss between the Caucasian (38%) and the African American (65%) populations.

Aim # 3. To test families unlinked to *BRCA1*, *BRCA2*, and loci on 8p12-22 for linkage to the *KAI1* gene, a metastases suppressor gene, and to analyze sporadic breast tumors for mutations in *KAI*.

3.3.1. Single-strand conformation polymorphism (SSCP). We will use SSCP to screen large numbers of samples for sporadic and germline mutations in the *KAI1* gene. PCR is carried out as outlined above in 10 µl reactions. Direct incorporation of a- ^{32}PdCTP is used. The PCR products are denatured and analyzed on MDE gels (AT Biochem) run at 6 watts for 16 hours at room temperature. Although promising novel methods of mutation screening such as the bacteriophage resolvase system are in the horizon, our experience indicates that SSCP is adequate for the rapid screening of large numbers of samples. We will not hesitate to employ a more accurate and sensitive method if one becomes available by the time this project gets underway.

3.3.2. Sequence of variant and normal DNA. After alignment of the autoradiographs with orientation markers to the gels, the variant SSCP bands are scraped directly into 50 µl PCR reactions as previously described (18). The DNA is amplified as above with 1 µl of 10 mM forward and reverse primer for 30 cycles. After verification of yield and purity on a 2% agarose gel, the DNA is purified on a G-50 spin column (5 prime-3 prime). Precipitation with ammonium acetate and glycogen and resuspension in 10 ml

TE, pH 8.0, 2 ml are used for cycle-sequencing (Stratagene *fmol*), as previously described (18). The gels are run at 1800 volts for 3-4 hours, dried, and exposed at room temperature for 16 hours.

3.3.3. Evaluation of LOH in tumor and normal samples. After the isolation of candidate genes at 8p, studies of somatic mutations and LOH experiments will be done concurrently. Although these experiments do not prove causality, the presence of both LOH of one allele and of a likely inactivating mutation of the other allele, strongly suggest that the tumor suppressor gene at that locus is implicated in the carcinogenic pathway. LOH will be tested on those tumors which exhibit mutations in *KAI1* metastases suppressor gene. Its importance in prostate cancer and the overlap between *BRCA1* and *BRCA2* and prostate cancer, suggest that it is reasonable to conduct the LOH experiments at 11p11.2 on other hormonally-dependent tumors such as breast and ovarian cancer. For those tumors with LOH at this locus, SSCP of *KAI1* will be conducted. Germline alterations will be tested on a panel of 100 normal chromosomes. Although no metastases suppressor gene has ever been described to be implicated in a familial breast cancer syndrome, the experiments on *KAI1* are motivated, in part, by recent mammographic and surgical data from the University of Michigan (Halvie, et al, submitted) suggesting that familial tumors may metastasize earlier than size-matched sporadic tumors. One (of many) possible explanation for these findings is that some of these families carry a germline mutation to a metastases-associated tumor suppressor gene, such as *KAI1* . These experiments are not very costly as *KAI1* is a small gene, but, if positive, have the potential of making very important original contributions to our thinking on breast carcinogenesis.

Statistical considerations We will analyze 100 minority and 100 Caucasian specimens and compare the African American and Caucasian groups for statistically significant differences in the frequency of deletions on chromosome 13q12-13 and 8p12-22 by the method of Chi-squares, Fisher exact test. We expect 80% power to detect 30% differences in frequency of deletions at any of these loci.

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Appendix

AIBS #349 - Defining the Smallest Common Region of Chromosome 17p that is Deleted in Sporadic Breast Tumors

Principal Investigator: Sofia D. Merajver, M.D.

Loss of heterozygosity (LOH) on the distal short arm of chromosome 17 is frequently observed in several different types of human tumors including colon (1), lung (2), ovary (3) and breast (4-7). In colon and lung tumors, the *p53* tumor suppressor gene located at 17p13.1 is the likely target of LOH. However several investigators have reported allelic loss at sites distal to *p53* in the absence of LOH at the *TP53* locus in both ovarian (3) and breast tumors (4-5). In addition, two groups have identified several breast tumors that demonstrate LOH at the *D17S5* or *D17S34* loci at 17p13.3 in the absence of mutations in the *p53* gene (7-8). These findings have led many investigators to propose that a second tumor suppressor gene exists on 17p13 distal to the *p53* gene, which is involved by LOH in 52-68% of sporadic breast tumors (5-8).

The polymorphic markers employed most frequently to identify LOH at distal 17p13, *D17S5* and *D17S34*, are located 20 cM and 28 cM, respectively, from *TP53* (9). Despite the publication of several genetic and physical maps of chromosome 17 in recent years (10-14), efforts aimed at narrowing this interval have been hindered by the relatively small number of highly polymorphic markers available from the candidate region. Isomura performed LOH with a panel of restriction fragment length polymorphisms (RFLPs) and reported that the smallest overlapping region (SOR) of LOH was flanked centromerically by *D17S878* and telomerically by *D17S34* (15). The authors estimated these two loci to be at least 7 Mb apart. Using a similar approach, Cornelis has defined the critical region as extending from *D17S5* to the telomere (8), a region spanning more than 6 cM. In order to isolate the critical gene (or genes) from this region a physical map of the region with densely spaced genetic markers must be assembled. We plan to approach this project using a unique approach utilizing a microdissection library from this region to isolate a large number of sequence-tagged sites, and to use these STSs to isolate cosmids from the chromosome 17 restricted cosmid library we have obtained from the Los Alamos National Laboratory. These cosmids will then be used as probes for fluorescent in situ hybridization (FISH) analysis of breast tumors. This approach allows us to define limits of the region of loss of heterozygosity without the need for polymorphic markers or normal DNA.

Construction of a 17p*ter* Microdissection Library

Microdissection of normal human G-banded metaphase chromosomes was carried out essentially as previously described (16). Briefly, normal metaphase spreads were prepared by standard cytogenetic techniques on glass coverslips and stained by trypsin Giemsa banding. Microdissection was performed with glass microneedles controlled by a micromanipulator mounted on an inverted microscope. Approximately 20 copies of 17p*ter* were scraped and the chromosome fragments adhering to the glass microneedles were transferred to a 20 ul collection drop (containing proteinase-K 50 ug/ml) in a 0.5 ml microcentrifuge tube. The recovered DNA was amplified by PCR using a degenerate oligonucleotide primer, UN1 (17), (5'-CCGACTCGAGNNNNNNATGTGG-3') at a concentration of 1.5 uM. The PCR reaction was performed in a 50 ul volume containing 200 uM of each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM TrisHCl pH8.4, 0.1 mg/ml gelatin and 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus). The reaction was overlayed with oil and heated to 94° C for 4 min and cycled for 8 cycles at 94° C for 1 min, 30° C for 1 min, and 72°C for 3 min followed by 28 cycles of the same conditions except for 56° C annealing temperature and a final extension of 72° C for 10 min. A portion of the amplified dissected DNA (100 ng) was labeled with biotin-11-dUTP in a secondary PCR reaction identical to the primary PCR reaction except the concentration of dTTP was decreased to 100 uM and biotin-11-dUTP was added at a concentration of 100 uM. Twelve cycles were performed with a 56°C annealing temperature. The biotinylated PCR product was

used as a probe for FISH on normal human metaphase chromosomes in order to confirm the regional authenticity of the microdissection. FISH was performed as described below.

A "microclone" library was constructed by performing a secondary PCR reaction on the original amplified, dissected DNA using primers designed with the first nucleotides complementary to the cloning site in the pAMP UDG cloning vector (Promega), and then followed by the first 10 nucleotides in the UN1 primer (sequence of UNU-A 5'-CUACUACUACUACCGACTCGAC-3', UNU-B 5'-CAUCAUCAUCAUCCGACTCGAC-3'). The PCR product was then directly ligated into the pAmp vector after treatment with uracil DNA glycosylas (UDG). Competent E. coli were transformed with the recombinant plasmids and bacterial colonies containing plasmids with inserts were identified by X-gal blue/white color selection.

Characterization of the Microclone Library

Microclones containing repetitive sequence were identified by filter hybridization with a probe containing human Cot-1 DNA labeled with ^{32}P-dCTP by the random primer method (18). Probes were purified by centrifugation in sephadex G-50 (Pharmacia Biotech) columns. Hybridizations were carried out at 65^0 C after overnight prehybridization in the presence of 6X SSC, 1% SDS, 1X Denhart's solution and 100 ug/ml of sheared salmon sperm DNA. Filters were washed for 30 min at 65^0 C in 2X SSC and 0.1% SDS. Autoradiograms were exposed overnight at -70^0 C. Any microclone hybridizing to the Cot-1 probe was removed from the library. The resulting single-copy sequence microclone library was analyzed for redundancy by gridding each individual clone onto nylon filters, then sequentially hybridizing each microclone with the gridded filters. The microclones were labeled with ^{32}P-dCTP by PCR utilizing the UNU-A and -B primers at a concentration of 1 uM. The labeling reaction was performed in 20 ul volumes with 1.5 mM Mg_2Cl, 200 uM of dATP, dTTP, dGTP and 50 uCi of α-^{32}P-dCTP (3000Ci/mmol), and 1 unit Taq polymerase (Boehringer Manheim). Thirty cycles were performed with the conditions described above with a 56^0 C annealing temperature. Probes were purified as described above, hybridization and washes were performed as described above.

FISH analysis of the microdissected material on normal human metaphase chromosomes confirmed that the recovered DNA originated from 17p*ter* . The microclone library contained two hundred microclones. Six of the microclones contained repetitive sequence by Cot-1 screening. Redundancy analysis resulted in the identification of 69 unique, single-copy sequence microclones.

Isolation of Region-Specific Cosmid Clones

Each unique, Cot-1-negative microclone was used as a probe to screen a flow-sorted chromosome 17 cosmid library which has been arrayed and stamped onto high density filters. The cosmid library contains approximately 16,000 clones gridded onto 11 filters. Microclones were labeled with ^{32}P-dCTP by PCR as described above. Hybridizations were carried out as described above. Filters were washed at room temperature for 30 min in 2X SSC and 0.1% SDS. Each cosmid identified was purified by streaking onto agar plates of "V broth" with Kanamycin. Four individual colonies were selected from each plate and stamped onto low density nylon filters which were re-screened with the corresponding microclone to verify the authentic positive cosmid clones.

Mapping Cosmid Clones by FISH

One cosmid isolated with each microclone was mapped by FISH on a mouse-human somatic cell hybrid panel composed of a chromosome 17-only hybrid cell line (MH 22-6) and 4 hybrid cell lines with deletions of various sizes at 17p13 (JW4, BR8, CA2 & AY1) which provide sublocalization within band 17p13 (20-21).

Two hundred and thirty-five cosmid clones were isolated initially utilizing the microclones as probes on the high density filters. One hundred and thirty-three cosmids were confirmed positive by re-screening with the low density filters of purified clones, representing thirty-four groups of non-overlapping cosmids. One cosmid from each non-overlapping group is now being mapped by FISH.

TABLE 1.

Characterization of 17pter Microdissection Library

Number of Microclones Picked	200
Cot-1 Negative Microclones	196
Unique Microclones	69
Cosmids initially identified	235
Cosmids confirmed on low dens filter	133
Non-overlapping groups of cosmids	34
Cosmids mapping to target region	?